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Low-Cost Fabrication of Centimetre-Scale Periodic Arrays of Single Plasmid DNA Molecules

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Abstract

We report development of a low-cost method to generate a centimetre-scale periodic array of single plasmid DNA of 11 kilobase pairs. The arrayed DNA is amenable to enzymatic and physical manipulation.

Introduction

Intermolecular heterogeneity of genomic DNA within a tissue or cell community commonly exists and is implicated in human health as exemplified by the multiple mutations of nuclear DNA within an individual tumor,^{1,2,3} heteroplasmy of mitochondrial DNA within not only a single tumor but also normal tissues,⁴ and numerous microbial genomes in human gut flora.⁵ Accurate, sensitive and comprehensive characterization of intermolecular heterogeneity requires the use of single-molecule assays such as the third-generation DNA sequencing techniques and DNA fiber assays.^{6,7,8} Central to these assays is the manipulation of single DNA molecules. A group of methods for manipulating single DNA molecules is featured by patterning single DNA molecules into a two-dimensional (2D) periodic array, a format that can facilitate high-throughput and highly parallel data acquisition and analysis.⁹

Several methods are able to prepare 2D periodic arrays of single DNA molecules. In one method, Levene *et al.* immobilized a single DNA polymerase within a nanohole to capture a single single-stranded DNA molecule.⁹ Similarly, Palma *et al.* placed streptavidin on 10–30 nm-wide metallic dots to capture single biotinylated oligonucleotides.¹⁰ However, fabrication of the nanoholes and nanodots requires electron-beam lithography (EBL), which is a serial, low-throughput nanofabrication technique and needs expensive instrumentation. Moreover, modification of the DNA increased complexity and cost of the method. In a different method, Josephs and Ye created 50 nm-wide features on a gold surface with scanning probe lithography (SPL) and captured single thiolated double stranded DNA of 94 base pairs with the nanofeatures.¹¹ SPL, however, is also an expensive and serial technique. Therefore, the EBL and SPL-based methods are unsuitable for large-scale production of

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single-DNA arrays over a centimetre-scale area. Alternatively, Plénat *et al.* used microcontact printing (μ CP) to create 800 nm-wide surface patches and immobilized single DNA-microbead complex on each patch.¹² μ CP allows parallel pattern generation and is routinely used to produce centimetre-scale microfeature arrays. Moreover, it is widely regarded as a low-cost fabrication technique. However, in this method for preparing DNA arrays, the DNA molecules needed to be attached to microbeads and multiple DNA molecules could be tethered to a single microbead. Moreover, the longest DNA patterned was ~2 kilobase pairs (kbp). Taken together, a low-cost, high-throughput fabrication method that allows generation of centimetre-scale periodic arrays of single unmodified DNA much longer than 2 kbp has not been reported. It should be noted that numerous commonly used or studied DNA molecules such as most plasmid DNA and human chromosomal DNA are much longer than 2 kbp in their intact form, so it is desirable that single-DNA arrays can be prepared with longer DNA molecules.

In this paper we introduce a novel μ CP-based method that allows for creation of ~200 nm-wide dot-like surface features on a glass surface and use of the nanofeatures for fabricating a centimetre-scale periodic array of single unmodified DNA molecules of 11 kbp. Note that conventional μ CP does not allow production of the ~200 nm-wide features. Although nanocontact printing (nCP), which uses a stamp with nanometer-scale surface structures can, in principle, generate the nanofeatures, other expensive techniques such as EBL are needed to create the template nanofeatures for preparing the stamp.^{13,14,15} It should also be noted that a special version of μ CP can create nanofeatures by controlling spreading of the printed material on gold,¹⁶ but gold is not desirable for many applications due to its lack of high optical transparency.

Results and discussion

Our method is schematically shown in Figure 1a. Briefly, a polydimethyl siloxane (PDMS) stamp bearing an array of circular micropillars (1 μ m diameter, 1 μ m height, and 2 μ m center-to-center distance, square lattice) was transiently dipped in an aqueous solution of sodium salt of polyacrylic acid (PAA) to form a PAA nanoparticle on each of the micropillars.¹⁷ The PAA nanoparticles were printed on to a glass slide coated with a thin layer of 3-aminopropyltriethoxysilane (APTES). The slide was then exposed to argon plasma, which removed the APTES only at the areas not covered by the PAA nanoparticles. The slide was washed with water to remove the PAA nanoparticles, generating an array of APTES nanofeatures called nanopatches here surrounded by bare glass surface. Figure 1b shows an AFM image of the APTES nanopatches with an average width of 196 nm [standard deviation (s.d.) = 20 nm, n = 24] and thickness of 2.4 nm (s.d. = 0.4 nm, n = 24). The size of the nanopatches can be controlled by adjusting the concentration of PAA as shown in Figure 1c. 1%, 3%, 5%, and 10% (wt/v) PAA solutions generated nanopatches with diameters of 98 nm (s.d. = 14 nm, n = 20), 186 nm (s.d. = 52 nm, n = 20), 240 nm (s.d. = 40 nm, n = 20), and 475 nm (s.d. = 9 nm, n = 20) respectively. While the nanopatch arrays could be routinely generated using 3%, 5%, and 10% solutions, it is of note that printing with 1% solution suffered from a relatively high percentage of missing nanoparticles in the printed array. Since transfer of the PAA nanoparticles from the stamp to the substrate was highly reliable for 3%, 5%, and 10% PAA solutions, we believed that PAA nanoparticles failed to form on some of the micropillars at 1% PAA concentration. 3% PAA solution was used to generate the APTES nanopatches of ~200 nm in diameter for preparing DNA arrays in this study. We further confirmed generation of the APTES nanopatch array by staining the APTES with fluorescein isothiocyanate (FITC), which can form covalent bond with primary amines and emits green fluorescence light. Figure 1d shows a portion of a 2 cm $\times$ 1.5 cm array of APTES nanopatches stained with FITC. Such an array was obtained routinely with a few scattered defects such as missing nanopatches.

Our μ CP method features an ability to produce a centimetre-scale array of nanopatches using a PDMS stamp bearing micrometer-sized surface features. Moreover, the method is simpler and less costly than other techniques that can fabricate similar nanofeatures including deep ultraviolet lithography (DUVL), EBL, polymer pen lithography (PPL), beam pen lithography (BPL), nanoimprint lithography (NIL) and nCP for the following reasons.^{13,18,19,20,21,22} First, the master for preparing the PDMS stamps can be produced by regular photolithography that is widely accessible and a master can be used to prepare many stamps. Second, the stamp is made of Sylgard 184 PDMS, which is the most commonly used material for preparing μ CP stamps. The stamp can be used multiple times for performing μ CP. Third, expensive instruments such as those used for DUVL, EBL, PPL, BPL and NIL are not required for preparing the stamp and for performing μ CP after a master is produced. Finally, our method is environmentally benign and cost effective due to the use of water to remove residual PAA nanoparticles in contrast to other methods in which organic solvents such as toluene and acetone are used for a similar purpose.²³

Critical to our method is the formation of the PAA nanoparticles on the micropillars. This process, which involves retention of a droplet of PAA solution on each micropillar, nucleation and growth of a PAA nanoparticle in each droplet, and evaporation of water from the droplet, currently is not fully controllable as evidenced by our inability to create an array of sub-100 nm patches with a low percentage of missing patches. However, this limitation may be overcome by elucidating the process of how the PAA nanoparticles form and optimizing the experimental conditions.

We prepared the single-DNA array by incubating an APTES nanopatch array in an aqueous solution of plasmid DNA. APTES is commonly grafted on glass to render the surface positively charged.^{23,24,25} Since the pK value of the primary amine of the surface grafted APTES is significantly lower than that in a bulk solution,^{26,27} DNA solutions with an acidic pH of 4.5 were used to prepare the DNA array. We assumed that the APTES nanopatches were positively charged in the DNA solution, allowing immobilization of unmodified DNA molecules by electrostatic interaction. Circular plasmid DNA of 11 kbp was used in this study as a model long DNA. The gyration diameter of the DNA should be between 234 and 338 nm when supercoiled, and between 304 and 386 nm when open-circular.²⁸ We hypothesized that a nanopatch with ~200 nm width was so small that its occupancy by a single 11 kbp DNA molecule would prevent a second plasmid from binding to this nanopatch due to electrostatic repulsion between the two DNA molecules.

Generation of the envisioned single-DNA array required that the DNA molecules be tightly controlled in size and isolated from one another in solution. We first confirmed the 11 kbp size of the DNA by gel electrophoresis (Figure S1a in Electronic Supplementary Information (ESI)). To further confirm that the DNA molecules were individually isolated in solution, we dropped the solution on a stationary featureless APTES-coated glass. Randomly scattered dots with uniform sizes and fluorescence intensities were obtained (Figure S1b in ESI), suggesting that each dot represented a single plasmid DNA. However, there existed a very small portion of dots with fluorescence intensities significantly lower than the majority of the dots. These dots could be fragments of the plasmid with much smaller sizes than the intact DNA. The fluorescence intensities of 222 individual dots were measured and plotted as a histogram (Figure S1c in ESI). The Gaussian fit revealed the peak center at 1071. To further examine the DNA solution, we used the spin-coating technique to stretch DNA and obtained rod-like structures (Figure S1d in ESI).^{29,30} The lengths of the rods were around 1.3 μ m. Given that a fully stretched 11 kbp circular DNA is 1.87 μ m long by assuming a 0.34 nm length per base pair, we believed that the rods were single partially stretched plasmid DNA molecules. Absence of much longer rods also suggested that the DNA

molecules in the solution were neither entangled nor aggregated. This result further supported that each fluorescent dot in Figure S1b was a single plasmid DNA molecule.

Next we sought to generate single plasmid DNA arrays on the APTES nanopatch arrays. A timed experiment was first performed over 90 min to monitor formation of the array. By defining array occupancy as percentage of occupied nanopatches over the total number of nanopatches,

Figures 2a1–2a4 clearly show an increase of array occupancy with time. Quantification of the array occupancy versus time is plotted in Figure 2b. The occupancy increased rapidly during the first 60 min and reached 90% at 90 min. To assess whether each nanopatch was occupied by a single plasmid DNA molecule, we monitored fluorescence intensities of 11 occupied nanopatches during the 90 min period with 4 of them being marked in Figures 2a1–2a4. The result is plotted in Figure 2b, showing a gradual decrease in average fluorescence throughout the 90 min time period, as expected due to bleaching during the imaging process. By assuming that binding of a second DNA to an occupied nanopatch would result in increased fluorescence of the nanopatch, we believed that each of the 11 nanopatches was only occupied by one plasmid DNA.

Figure 2c shows a portion of a 2 cm × 1.5 cm DNA array with ~96% of all nanopatches being occupied by the DNA. We measured fluorescence intensity of each fluorescent nanopatch of the micrograph and performed a Gaussian fit (Figure 2d), showing the peak center at 1014, which was comparable to that of the DNA immobilized on the featureless APTES-coated surface. This result further suggested that each nanopatch was occupied by a single plasmid DNA molecule.

To further confirm the single-DNA occupancy of the nanopatches, we generated DNA arrays using the plasmid DNA labeled with fluorophores of two different colors (red and green). In this method, two aliquots of the DNA were labeled with rhodamine and YOYO-1 respectively. The two aliquots of labeled DNA were mixed at a 1:1 mole ratio and immediately applied on the nanopatches to form the DNA array. A nanopatch occupied by both red and green DNA molecules would appear yellow in an overlaid fluorescence micrograph. If the single-DNA occupancy was strictly attained, half of the occupied nanopatches would be red and the other half green. If every nanopatch was occupied by two DNA molecules, 50%, 25% and 25% of the occupied nanopatches would be yellow, red and green respectively. Presence of a very low percentage of yellow dots in an array with a high array occupancy would thus indicate achievement of a high degree of single-DNA occupancy per nanopatch.

In order to establish the above method, we first needed to confirm that this DNA labeling method did not significantly fragment or crosslink the DNA and that single labeled DNA molecules were visible under our fluorescence microscope. Gel electrophoresis of the labeled DNA (Figure S2a in ESI) showed that the DNA remained largely intact. Fluorescence micrograph of the labeled DNA immobilized on an APTES-coated featureless surface (Figure S2b in ESI) revealed that the labeled DNA molecules were well dispersed in solution and that single rhodamine-labeled DNA molecules were visible. As the plasmid stained with YOYO-1, there also existed a very small portion of dots significantly fainter than the majority of the dots. These dots could be plasmid fragments much smaller than 11 kbp. Moreover, the DNA stretched on a spinning APTES-coated featureless surface displayed a rod-like shape with lengths around 1.3 μm (Figure S2c in ESI), further indicated that the labeling procedure did not affect the structure and crosslink the DNA in solution.

Figure 3a shows an array prepared using the mixed red and green plasmid DNA with an array occupancy of 91%. Eleven dots were yellow in this micrograph, accounting for 1.5%

of all occupied nanopatches. Analyzing five $110\ \mu\text{m} \times 110\ \mu\text{m}$ micrographs that contained ~15,125 nanopatches demonstrated an overall 87.3% array occupancy with 3.1% yellow dots. This low percentage of the yellow dots indicated that vast majority of the nanopatches were occupied by single DNA molecules. Occupancy of a nanopatch by more than one molecule could be attributed to the DNA fragments that probably existed in the DNA solution. Some fragments might have much smaller sizes than that of the intact DNA. Occupancy of a nanopatch by such a fragment might have allowed binding of the nanopatch by a second DNA molecule. As a control, we prepared $1\ \mu\text{m}$ -diameter APTES patches and found that majority of the patches allowed occupancy by multiple DNA molecules (Figure 3b), indicating that $1\ \mu\text{m}$ diameter was too large to restrict occupancy of a patch to a single plasmid DNA of 11 kbp.

The single-DNA array developed here is intended to be used for biomedical assays. DNA-protein interaction plays a critical role in many of the assays as exemplified by the polymerase-DNA interaction used for sequencing single DNA molecules.⁹ However, it is reasonable to speculate that the strong electrostatic attraction between the immobilized DNA and APTES nanopatches may hinder the DNA-protein interaction. To assess this speculation, a preformed array of the DNA labeled with YOYO-1 dye was exposed to DNaseI. As shown in Figure 4a, DNaseI exposure resulted in disappearance of the fluorescent dots in the array. In contrast, a DNA array exposed to inactivated DNaseI remained unchanged (Figure 4b). Moreover, a new DNA array could be formed on the array substrate after stripping off the previously immobilized DNA with DNaseI (Figure 4c). These results indicated that the immobilized DNA molecules were enzymatically degraded by DNaseI and suggested that the periodic single-DNA array might be processable by other enzymes such as polymerase for sequencing the single long (11 kbp) plasmid DNA molecules in a massively parallel way.

DNA fiber assays rely on stretching DNA molecules into an elongated form.^{7,8} While periodic arrays of stretched λ -DNA have been produced,^{31,32} generation of a dense array of circular plasmid DNA with a much shorter length than λ -DNA has not been reported. The plasmid DNA molecules immobilized on the APTES nanopatches were obviously condensed as compared to their stretched counterparts in Figures S1d and S2c in ESI. Since electrostatic interaction was probably responsible for the DNA condensation, we hypothesized that a condensed DNA molecule could be partially released by reducing the electrostatic interaction and the partially released DNA could be stretched on the substrate surface. PAA, the polyelectrolyte used for preparing the APTES nanopatches in this study, was added on a preformed DNA array for this purpose due to its anionic nature. The partially released DNA was stretched with a dewetting meniscus generated by pulling the array substrate out of an aqueous solution at a controlled speed. Tween 20, a nonionic surfactant, was added in the PAA solution and found to be essential for dewetting to occur on the hydrophilic surface of the glass slide. The result (Figure 4d) shows a periodic array of rod-shaped structures with lengths (average = $1.3\ \mu\text{m}$, s.d. = $0.4\ \mu\text{m}$, $n = 221$) similar to those of the DNA stretched on the featureless APTES-coated surfaces (Figures S1d and S2c in ESI), strongly suggesting that the rods were stretched single plasmid DNA molecules. The stretched single DNA array may allow performing high-throughput DNA fiber assays for various applications such as characterizing mutations in human mitochondrial DNA (mtDNA). Human mtDNA is circular and has a size of 16.5 kbp, which is comparable to that of the plasmid used in this study. Moreover, a 4977 bp deletion in mtDNA is strongly correlated with cancer and ageing.³³ Our technique promises to generate arrays of stretched single mtDNA molecules and realize high-throughput characterization of the deletion by one of the fiber assays – DNA fluorescent *in situ* hybridization.⁸

Conclusions

We have developed a low-cost μ CP method for fabricating a centimetre-scale periodic array of positively charged nanofeatures called nanopatches. The nanopatches allowed immobilization of unmodified DNA molecules and largely restricted the occupancy of one 200 nm-wide nanopatch to a single plasmid DNA molecule of 11 kilobase pairs, so that a single-DNA array was produced. The immobilized DNA was amenable to enzymatic and physical manipulation, suggesting that this novel single-DNA array can be used for various biomedical assays such as sequencing of single DNA molecules and fiber assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Fabrication and characterization of nanopatches. PAA: sodium salt of polyacrylic acid. APTES: 3-aminopropyltriethoxysilane. (a) Schematic diagram of the process for generating a centimetre-wide array of single plasmid DNA. (b) Atomic force microscopy image of 196 nm-diameter APTES nanopatches on glass. (c) Plot of nanopatch size versus PAA concentration. (d) Fluorescence micrograph of APTES nanopatches stained with fluorescein isothiocyanate.

Figure 2.

Characterization of the single-DNA array. (a1–a4) A series of fluorescence micrographs of the same area of a DNA array recorded at time points of 5, 30, 60, and 90 min. Arrowheads point 4 nanopatches that were monitored throughout this period of time. (b) Plot of array occupancy versus time (red line) and time-lapse fluorescence intensities of 11 selected nanopatches (blue line). Dots represent averages and bars represent standard deviations. $n = 3$ (red) and 11 (blue). The gradual decrease in fluorescence resulted from bleaching of fluorescence during viewing. (c) Fluorescence micrograph of a DNA array with a high array occupancy. (d) Histogram of fluorescence intensity of 500 nanopatches occupied by DNA in an array with a Gaussian fit (red line, center of peak: 1014). The DNA was labeled with YOYO-1 dye.

Figure 3. Two-color DNA arrays formed on nanopatches and micropatches. Overlaid fluorescence micrographs of plasmid DNA labeled with rhodamine (red) and YOYO-1 (green) respectively immobilized on (a) nanopatches and (b) 1 μm -diameter micropatches. Colocalization of YOYO-1-labeled and rhodamine-labeled DNA molecules yielded yellow color. Yellow dots in (a) are marked by arrowheads.

Figure 4.

Enzymatic and physical manipulation of DNA in the single-DNA arrays. Fluorescence micrographs of (a) a DNA array after being treated with DNaseI, (b) a DNA array after being treated with inactivated DNaseI, (c) a DNA array that was first treated with DNaseI, washed, then regenerated by adding the DNA, and (d) an array of stretched DNA. The DNA was labeled with YOYO-1 dye.